

# Inflammatory aortic abdominal aneurysm – immunophenotypic characterization of inflammatory infiltrate

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Abdominal aortic aneurysm (AAAs) constitute an important clinical problem as they occur in up to 8% of male patients over 60 years of age, and their complications such as aneurysm rupture are associated with 80% mortality [1]. While the pathogenesis and epidemiology of classical AAAs are relatively well studied, inflammatory aneurysms (IAAA) create a particularly interesting subset which represents 5% to 10% of AAAs and are characterized by a firm, thick aortic wall with a shiny white appearance and peri-aneurysmal infiltration or retroperitoneal fibrosis which can be detected by computed tomography (CT) scan or ultrasonography (USG), as a cuff of soft tissue inflammation surrounding the aneurysm [2, 3]. Their development involves a predominant immune response within the vessel wall [2], although its nature remains poorly defined. Inflammatory markers (C-reactive protein (CRP) or leukocytes (WBC)) are elevated [2]. The inflammatory process leads to more complicated IAAA repair and subsequent post-operative period.

There are a number of differences in the epidemiology and clinical presentation of IAAA. Inflammatory abdominal aortic aneurysm AAAs are generally larger than AAAs [4]. The risk factors, such as male gender and smoking, have even stronger effects in relation to IAAA than for typical atherosclerotic AAA [4, 5]. The male : female ratio is almost 5-fold higher than for atherosclerotic aneurysm (30 : 1 vs. 6 : 1) [4, 5]. Although almost 90% of IAAA patients have symptoms such as abdominal or back pain, weight loss or elevated inflammatory markers, such as erythrocyte sedimentation rate, C-reactive protein or white blood cell level [6], these are nonspecific, which makes the diagnosis of IAAA difficult. Computed tomography (CT) scan and ultrasonography (USG) are useful and reliable imaging methods used for diagnosis of both inflammatory and atherosclerosis aneurysm. In this article we present an immunophenotyping method which might be useful for IAAA diagnosis and confirmation.

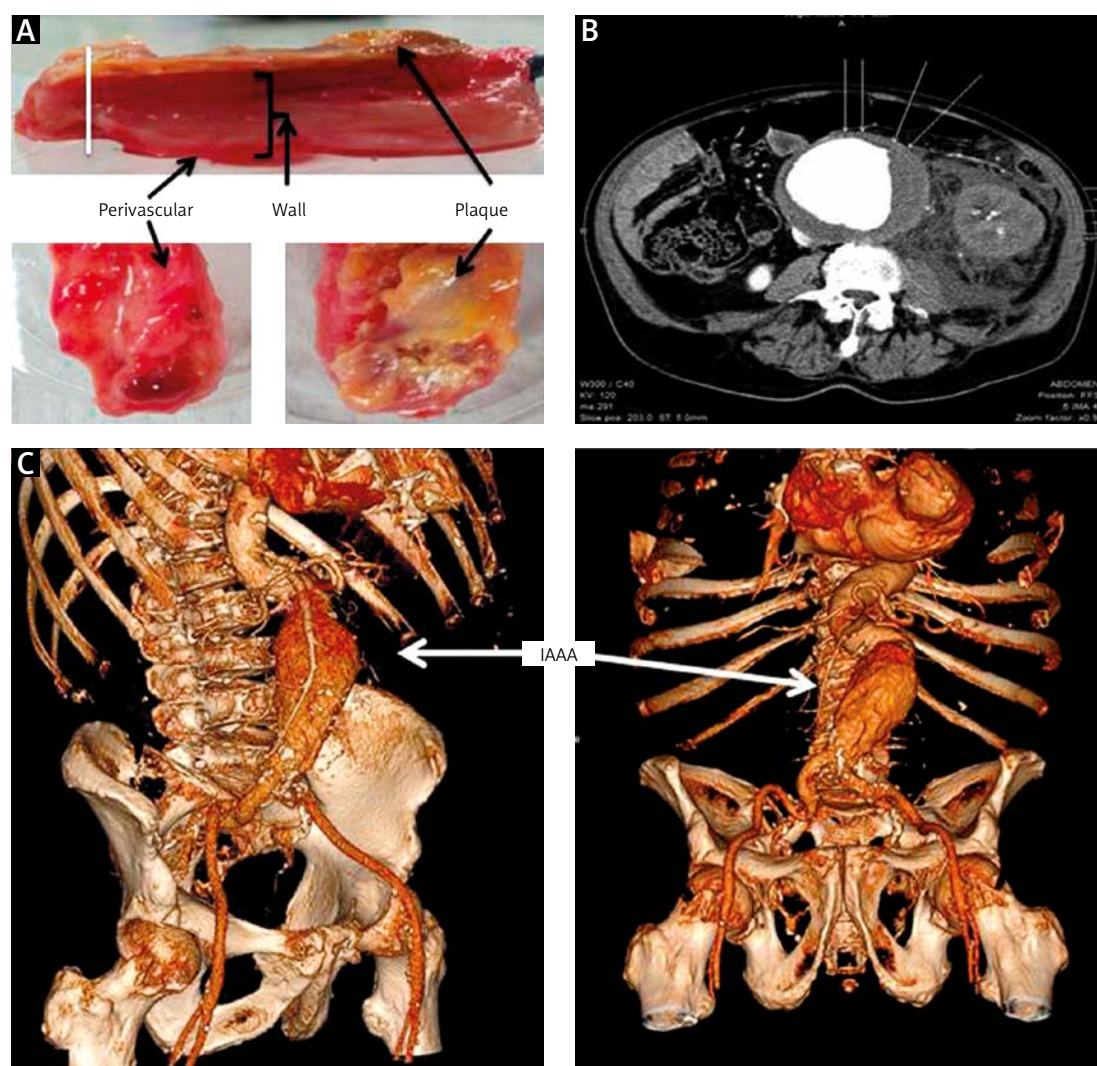
We studied IAAA wall inflammatory infiltrate obtained from a 60-year-old man, with significant obesity (body mass index (BMI) 30 kg/m<sup>2</sup>), in whom AAA was diagnosed 24 months prior to surgical repair. The IAAA diameter increased in this time from 4 to 6 cm. Smoking history includes 42 pack-years but was stopped 3 months prior to surgery. Diffuse coronary artery disease was present with myocardial infarction

(1995) and percutaneous coronary intervention (PCI) (2013) for which they received aspirin, clopidogrel, atorvastatin, bisoprolol and ramipril. Ankle brachial index was 0.9. Computed tomography demonstrated a clearly thickened wall of the abdominal aorta suggestive of IAAA (Figure 1 B), but the increase of inflammatory parameters was very modest (CRP 5.2 mg/l and WBC 6800/ $\mu$ l). During surgical repair the aneurysm wall was hard, “ivory” colored and thickened (100 mm), and was embedded in the surrounding infiltrate. After clamping the aorta, the aneurysm was repaired using a simple aneurysm graft. Upon releasing the clamp, no pulse was detected in the left groin, and an additional prosthesis between the aneurysm graft and common femoral artery was implanted. The postoperative course was complicated by a febrile (39°C) state, which subsided after 4 days, and the patient was discharged in good condition.

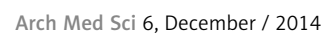
A fragment of the wall of the aneurysm was obtained at the site of maximal dilatation. After harvesting, the sample was placed in ice cold (4°C) phosphate buffered saline (PBS, Gibco, Invitrogen, Carlsbad, CA, USA). Additionally, before the surgery, a blood sample was collected in EDTA containing tubes (Becton Dickinson) from peripheral access for the determination of reference immunophenotype. Samples were transported to the laboratory immediately.

The Local Research Ethics Committee approved sample collection, written informed consent was obtained.

Peripheral blood was lysed using RBC Lysis Buffer (eBioscience, San Diego, CA, USA) (8 min, room temperature) and was washed three times with ice-cold PBS. Cells were incubated with fluorescently labeled antibodies anti-CD45 PeCy7, anti-CD3 PerCP, anti-CD4 APC, anti-CD8 APC-H7, as well as anti-CD25 PE, anti-CD69 FITC, anti-CD28



**Figure 1.** A – IAAA wall in the context of perivascular tissues and atherosclerotic plaque. The size bar indicates 15 mm. B – Angio-CT image showing an abdominal aorta aneurysm. Arrows mark lines showing the thickened and inflamed wall of the abdominal aorta. C – 3D reconstruction of IAAA (white arrows)



APC and anti-CCR5 PE (BD Biosciences) for 20 min at 4°C. After washing, cells were re-suspended in PBS with 1% fetal bovine serum (FBS) (Gibco), studied on a FACSVerse (BD Biosciences) and analyzed using FlowJo software.

Atherosclerotic plaque and perivascular tissue were separated from the wall (Figure 1 A). The wall was delicately mechanically disrupted and digested with a mix of digestion enzymes, collagenases and hyaluronidase in PBS with calcium-magnesium containing 20 mM HEPES, at 37°C, for 20 min, with gentle agitation to isolate residual cells infiltrating the wall. The resulting cell suspension was passed through a 70-µm strainer (BD Pharmingen, San Diego, CA, USA). Cells were incubated with the same fluorescently labeled antibodies using the same labeling procedures as were applied for blood.

The initial selection of blood cells was performed on FSC/SSC scatter, allowing CD45 antigen to be applied for leukocyte confirmation. Subsequently T cells (CD3+ positive) and their subpopulations, CD4 and CD8 positive, were identified (Figure 2 A). Additionally we studied the presence of antigens on T cells, such as CD25, CD69, CD28 and CCR5 (Figure 2 C). To study activation markers on T cells we used the isotype control (KI) or fluorescent minus one (FMO) method.

As for blood, tissue cells were first gated on FSC/SSC scatter and then CD45-positive cells were identified. T cells, their subpopulations and characteristics were identified using the same markers as for blood (Figures 2 A, C).

The aneurysmal wall was significantly infiltrated by leukocytes which importantly differed from peripheral blood. In particular, T cell infiltration was strongly increased in IAAA, constituting 68% of infiltrating leukocytes. These were predominantly CD4+ lymphocytes (Figures 2 A–B). Both CD4+ and CD8+ T cells were activated in the IAAA wall and expressed the early activation marker (CD69) (30% in IAAA vs. 1% in blood). CD25 (typically late activation) was increased only on CD8+ cells. In line with this, > 50% of CD8+ T cells in blood of the patient showed the senescent phenotype (lack of CD28 marker – CD8+CD28null). This population rarely exceeds 30–40% in healthy individuals. In contrast, CD4+<sup>CD28null</sup> T cells, characteristic for atherosclerotic plaques, were not found in the IAAA wall. T cells showed in turn high expression of the CCR5 receptor for the chemokine RANTES, which could in part explain high T cell recruitment (Figure 2 C).

In the IAAA patient described here, the clinical presentation included only abdominal pain from the usual (80%) triad including abdominal/lumbar pain, severe weight loss and elevated CRP [2]. Despite this, during surgery we observed a characteristic macroscopic view of IAAA with strong

peri-aortic infiltration, consistent with the classic Walker description [3, 7, 8]. Importantly, angio-CT could indicate the inflammatory nature of the AAA process. Therefore it should be kept in mind that diagnostic imaging is a very important tool, which can suggest pathology within the vascular wall and may guide optimal patient management and help predict possible complications [9]. Interestingly, the imaging technique was a more reliable indication of inflammatory nature than routine blood tests (CRP, WBC). This may be due to the local nature of inflammation or treatment with concomitant medications of an anti-inflammatory nature: statins/ACE-inhibitor/NSAID [8]. Interestingly, the patient developed peri-operative fever, which was not associated with infection and could be related to the inflammatory nature of the aneurysm. Recent studies highlight the possible role of infections in initiating IAAA [2, 10]. Presence of senescent CD8+ T cells could point towards a role of viral infection or an occult endogenous auto-immune process. While in the blood of IAAA patients we observed increased granulocytes and decreased total mononuclear cells when compared to values reported for atherosclerotic AAA blood, the IAAA wall was heavily infiltrated by leukocytes (ca. 80% of stromal fraction, vs. 40% in atherosclerotic AAAs; unpublished) which were predominantly activated T lymphocytes. Moreover, the presented case brings attention to the senescent CD8+<sup>CD28null</sup> cells as players in IAAA. These cells have recently been described in human hypertension. Our study may show that the chemokine RANTES, overproduced in IAAA, may play an important role in inflammation and thus may constitute a future important target.

In conclusion, in spite of the lack of a typical high rise of inflammatory markers (CRP, WBC), IAAA wall inflammation is characterized by activation of T cells, with a possible role of cytotoxic CD8+ cells, which have a senescent phenotype. This infiltrate is better reflected by the immunophenotypic method than typical inflammatory markers such as CRP or WBC.

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